NASA-CR-194325

8P

FINAL TECHNICAL REPORT NAG 2-410

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23 January, 1991

Period covered: 1 Jan 90-31 Dec 1990

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(NASA-CR-194325) FINAL TECHNICAL REPORT, 1 JANUARY - 31 DECEMBER 1990 (San Jose State Univ.) 8 p

N94-70586

Unclas

Z9/52 0185570

This is a summary report dealing with the results of research conducted on skeletal muscle atrophy during the past year. The research focused on two aspects of this problem: 1) two dimensional polyacrylamide gel electrophoresis of muscle tissue with the view of identifying proteins (other than contractile proteins) which appear to be associated with the atrophic process which unloaded muscle typically undergoes, 2) analysis of the activity of several proteolytic enzymes representative of the lysosomal, cytosolic and nuclear cell compartments of skeletal muscle with respect to their potential involvement in the accelerated breakdown of protein which occurs in atrophy in association with decreased protein synthesis. The muscles studied were obtained from rats whose hindlimbs were unloaded, from control rats, and from rats flown in the US-USSR Cosmos flights 1887 and 2044.

The results which were obtained are summarized in the following paragraphs.

1) Two dimensional electrophoresis (2D-PAGE): The expression of myosin light chains was altered from exposure to the microgravity of the Cosmos 2044 flight of two weeks duration. The proportion of fast myosin light chains increased at the expense of the slow myosin light chains in the adductor longus hindlimb muscle. One of the isoforms of the slow myosin light chains ($MLC1_{sa}$) was found to be a remarkably sensitive indicator of slow twitch oxidative fiber atrophy. At the present time this isoform is detectable only by 2D-PAGE. This isoform is also present in the soleus muscle, a slow twitch oxidative muscle, and is sharply depleted by hindlimb unloading.

The identity of the $MLC1_{sa}$ was confirmed by isolating it from transblots of 2D-PAGE gels, cleaving the isolated peptide with CNBr since the amino terminus was found to be blocked, and isolating several of the internal peptide fragments for microsequencing. The following sequences were found in three fragments:

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     1       10        20
1. M/RALGQNPTNAEVLKVLGNPK

2. M/GAELRHVLLTL(G)xK(M)

3. M/LQxVAKx(V)DQGxYED...
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These sequences show an 89% identity with the corresponding sequences of the human myosin light chain $1_{sa}$ recently determined from the cDNA clone (Hailstones and Gunning, 1990, Molecular Cell. Biol. 1o: 1095). Their nucleotide sequences and deduced amino acid sequences as well as our own directly determined amino acid sequence are shown in Fig.1. The reason for the preferential depletion of this MLC isoform in response to unloading is unknown and presents opportunity for the investigation of transcriptional and translational regulatory mechanisms in atrophy.

It is of interest that the amino terminal region of $MLC1_{sa}$ and $MLC1_{s}$ are rich in proline and alanine (50% of the first 51 residues). The reason for this is unknown, but may relate to folding of myosin as seems to be the case for smooth muscle myosin where an intact 20 kD light chain was found to be required for optimal folding at the hinge regions of the myosin rod (Trybus and Lowey, 1988, J. Biol. Chem. 263: 16485).

Another cytosolic protein whose concentration was found to diminish drastically after hindlimb unloading and after the Cosmos 2044 spaceflight was the fatty acid binding protein (MW=14,000). As revealed by 2D-PAGE this protein is present in high concentration in slow twitch oxidative muscle (32ug per mg protein), but is low in fast twitch muscle (3 ug per mg protein), (Miller et al. Proc. Soc. Exp. Biol. Med. 189: 183). Furthermore, immunohistochemical analysis has shown that the protein is almost exclusively localized in the oxidative fibers with only trace amounts in fast glycolytic fibers (Nielson et al. 1990 Mol. Cell. Biochem. 98:119). We found a marked reduction in the binding protein of the slow adductor longus muscle from rats that had undergone the Cosmos 2044 spaceflight or hindlimb unloading. The identity of this spot was confirmed by microsequencing the protein on transblots from 2D-PAGE. This protein also had a blocked amino terminus, so that CNBr was used to generate a peptide which contained 14 amino acids in the following sequence: M/KSLGVGFATRQVAS (M). This sequence was found to be unique to the fatty acid binding protein found in red and heart muscle of the rat (Claffey, K.P. et al. 1987, Biochemistry 26: 7900). The identification had a certainty of 98%. With the recent availability of an antiserum quantitative studies will be greatly facilitated.

The significance of the decrement in the fatty acid binding protein is indicative of the metabolic changes that are being sustained by the slow oxidative muscle fibers in the conversion to a higher population of fast glycolytic fibers. Thus, the increase of myosin light chains of the fast type, the disappearance of the $MLC1_{sa}$ isoform, and the reduced quantity of the fatty acid binding protein seem to form part of a pattern of biochemical alterations that constitute the response to altered functional demand on muscle i.e., unloading in microgravity.

Several other cytosolic polypeptides have been shown by 2D-PAGE to be significantly altered in concentration. Their partial sequences have been determined but are not in current protein sequence data bases so that their identity is unknown at present. For example a 19,000MW polypeptide (MSN# 68) contained a peptide obtained by CNBr cleavage that had the following unique sequence: (M/xIRVPVQSxLRRASAPLPGFS...). Since each month about 1000new peptide sequences are entered into data banks, it should not be too long before identity will be established.

2. Proteolytic Enzymes: Tripeptidyl peptide hydrolase (TPH) was found from earlier studies to be significantly elevated (59%) in atrophying soleus muscle taken from rats that had undergone a 7 day Spacelab 3 flight, whereas the EDL sustained only a marginal increase. This protease is generally localized in lysosomal membranes but has also been found in the sarcoplasmic reticulum of skeletal muscle (Riley et al., unpublished). Its exact role in muscle breakdown is not clear nor is it known if it hydrolyzes only specific proteins or acts indiscriminately on all proteins having accessible peptide bonds that meet the specificity requirements of the protease. We have developed an improved method for isolating the protease from isolated spleen or liver lysosomes (rich sources compared to muscle) by column chromatographic techniques. Fig. 2 illustrates a profile obtained from a C18 reverse phase column (TFA-acetonitrile solvent) of the highly purified enzyme prepared by phenyl Sepharose chromatography. In order to microsequence the enzyme the large peak will supply protein for sequencing because it is of quite adequate purity.

3.Assay of Proteases in Hindlimb Muscles of Rats from Cosmos 2044: A previous study of the content of muscle proteases from the 7 day Spacelab 3 flight showed an increase in TPH activity (Riley et al. 1987 Muscle & Nerve 10:560). The flight soleus showed a 59% increase in TPH activity and a 26% increase in the net $Ca^{2+}$ -activated protease activity (e.g. now named calpain). Significant differences between flight and simulation controls were not found for three other proteases of the dipeptidyl peptide hydrolase(DPH)family: Lysosomal DPH II, cytosolic DPH III, and microsomal DPH IV. However, the lack of signficant change in the activity of these enzymes may also mean that their activities may be regulated by changes of endogenous inhibitor or cofactor activities. These studies should be supplemented by immunochemical assay of enzyme protein concentration, since a large part of the activity may be suppressed, as for example, in the case of calpain where as much as 90% of the protease activity may be inhibited by the endogenous peptide inhibitor calpastatin.

During the past year the plantaris and EDL muscles of rats from the Cosmos 2044 flight (14 day duration) were made available for analysis. This included muscles from rats that served as unloaded hindlimb controls, sychronous controls, and vivarium controls. The muscles were assayed for additional protease activities: multicatalytic protease, calpain, TPH and DPH IV. These muscles are not particularly prone to atrophy from unloading or microgravity because they contain much lower percentages of the slow-twitch oxidative fibers which are especially sensitive to atrophy due to unloading. The soleus contains 75% slow oxidative fibers (SO) and 25% fast oxidative-glycolytic (FOG) fibers, whereas the EDL contains 10%, 66% and 24% of the SO, FOG, FG types, respectively (Riley et al. 1990 FASEB. J. 4: 84). Although the SO fibers are most prone to atrophy, the FOG and FG fibers also atrophy as indicated by the reduction in cross-sectional area on exposure to microgravity. On the average, flight soleus fibers decreased 38%, plantaris fibers by 21% and EDL fibers by 17.5% (Cosmos 1887, 12.5 days duration).

The plantaris and EDL muscles were assayed for the protease activities in 10% aqueous homogenates of the muscles using the following substrates and pH optima for the respective proteases:

| | | | |
|---|---|---|---|
| 1. | Multicatalytic protease (MCP) | Succ-Leu-Leu-Val-Tyr-AMC | pH 8 |
| 2. | Free Calpain (CAP) | Succ-Leu-Tyr-AMC | pH 7 |
| 3. | Tripeptidyl peptide hydrolase | Ala-Ala-Phe-AMC | pH 4 |
| 4. | Dipeptidyl peptide hydrolase | Gly-Pro-AMC | pH 8 |

The assay results are summarized in Table 1 except for DPH IV which is still in progress. Free calpain activity and TPH increased significantly in the EDL and plantaris muscles of the flight and hindlimb suspension (tail) groups. This is in contrast to the Spacelab 3 results which showed a small but insignificant change in these activities of the EDL muscle, even though the average cross-sectional area of the fibers of the EDL was reduced to the same extent. The greater proteolytic activities of the EDL are likely due to the longer flight duration of 14 days as compared to the 7 days in the Spacelab 3.

No change in the multicatalytic protease activity was detectable in either of the two muscles from any of the groups of rats. This would suggest that this complex of proteases does not play a major role in the general breakdown of protein in atrophy. It should be noted that the protease appears to be present in muscle cell nuclei as well as in the cytosol. This seems to be unique to muscle nuclei since other tissues do not show such localization (Stauber, W.T., 1987, Histochemical J., 19: 594). Assays for the nuclear content of this protease are in progress.

## Publications


1. Skeletal muscle fiber, nerve and blood vessel breakdown in spaceflown rats. Riley, D.A., E.I. Ilyina-Kakueva, S. Ellis, J.L.W. Bain, G.R. Slocum and F.R. Sedlak. FASEB J. 4:84-91; 1990

2. Cosmos 2044 Muscle Studies (Experiment K9-07). Final Science Report Ellis, S., Riley, D.A. and C.S. Giometti. NASA Technical Publication, in press.

3. Eccentric contraction-like lesions and disrupted microvasculature in rat skeletal muscle after spaceflight and hindlimb unloading. Riley, D.A., S. Ellis, C.S. Giometti. E.I. Ilyina-Kakueva, V.S. Oganov, G.R. Slocum, J.L.W. Bain, and F.R. Sedlak. J. Appl Physiol., in press.

## Table 1

### Summary of Protease Assays on Rat Plantaris and EDL

| Muscle/ Group | TPH | Free Calpain | Multicatalytic Protease |
|---|---|---|---|
| | Mean F.U. $min^{-1}$ ml. ± S.D. | | |
| PLANTARIS | | | |
| Flight | 1855 ± 125 | 63* ± 5 | 1525 ± 98 |
| Tail | 1900 ± 120 | 58* ± 7 | 1460 ± 160 |
| Syn | 1690 ± 162 | 45 ± 4 | 1365 ± 103 |
| Basal | 1665 ± 105 | 40 ± 5 | 1320 ± 112 |
| Vivar. | 1680 ± 175 | 42 ± 5 | 1475 ± 90 |
| EDL | | | |
| Flight | 1365* ± 105 | 86* ± 6 | 1355 ± 130 |
| Tail | 1460* ± 92 | 81* ± 4 | 1610 ± 188 |
| Syn | 1190 ± 80 | 65 ± 10 | 1480 ± 100 |
| Basal | 1140 ± 77 | 54 ± 8 | 1530 ± 96 |
| Vivar. | 1100 ± 82 | 68 ± 9 | 1425 ± 101 |

* $p \leq 0.05$ (t test) vs.pooled Syn, Basal, Vivar.; 5 muscles per group.

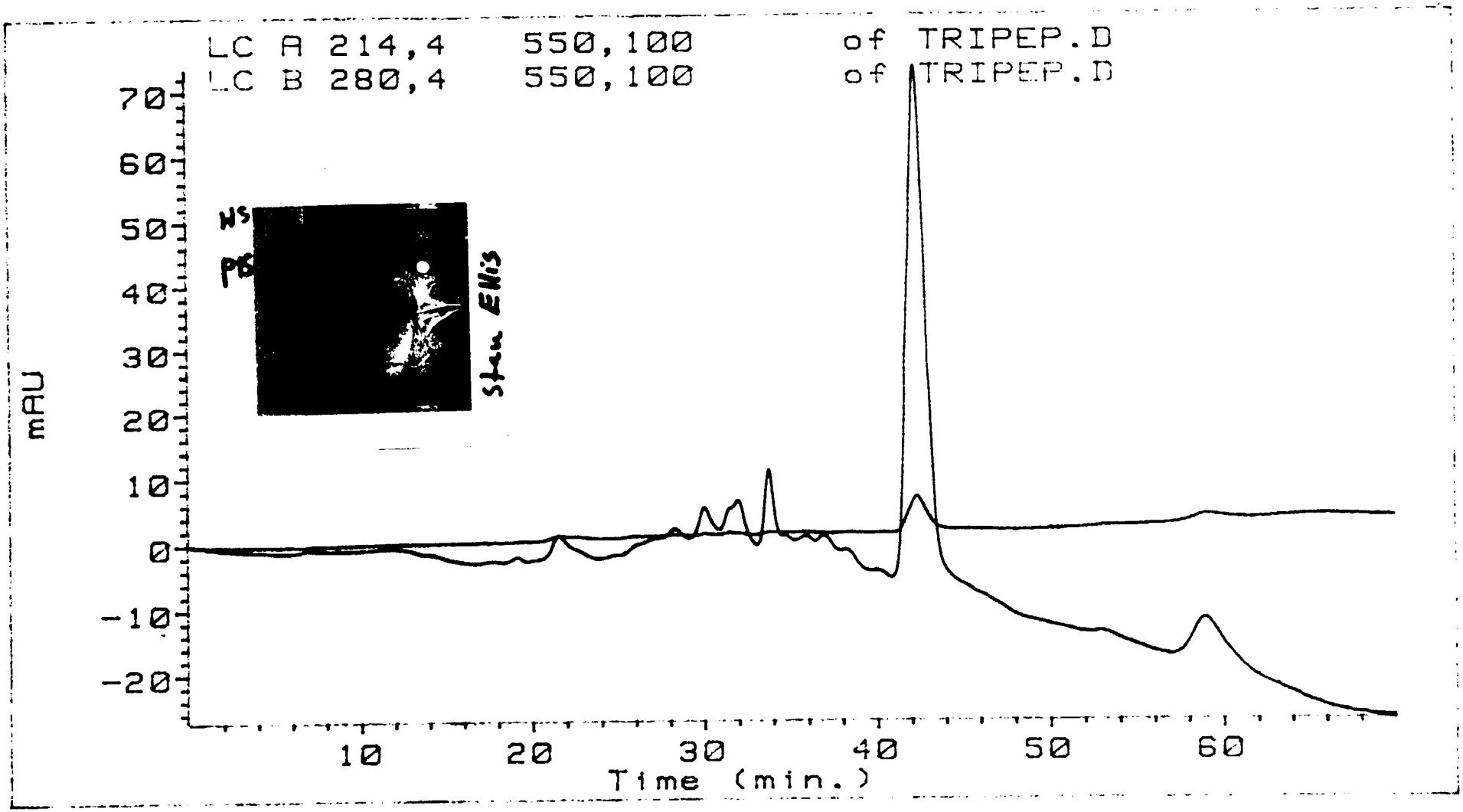
LC A 214,4 550,100 of TRIPEP.D
LC B 280,4 550,100 of TRIPEP.D
mAU
70
60
50
40
30
20
10
0
-10
-20
10
20
30
40
50
60
Time (min.)
NS
PB
Stan Ellis

FIG. 1